A CHARACTERIZATION OF COMPONENT VI, A LIPOPROTEIN, FOUND IN THE BLOOD SERUM OF DIETHYLSTILBESTROL TREATED BIRDS

by

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INTRODUCTION

The lipoproteins of serum consist of a peptide component in combination with lipid. This lipid includes phospholipid, cholesterol, cholesterol esters, and triglycerides. Due to the interest in the role of lipoproteins in lipid transport and their role in relationship to disease, much work has been done on human serum lipoproteins.

Previously it was thought that the lipid and protein moiety were held mainly by hydrophobic bonding; however, recent work has revealed that a salt linkage possibly plays a larger role. These linkages involve the free -COOH group of the dicarboxylic acid residues of the protein and phospholipid.

Component 6 is a lipoprotein entity present in the serum of the laying hen. It is also present in the serum of the estrogenized rooster or young pullet, and is not found in the serum of the non-estrogenized rooster or young non-laying hen (41). This entity has a very low density, and contains great quantities of lipid and a very small amount of protein. The protein is essential for the solubility of the lipid.

In an attempt to find the relationship of lipid to protein, differential lipid extractions were performed. The quantitative relationship of the different lipid classes and protein were also determined. These lipid studies were carried out by means of thin layer chromatography and various chemical and physical techniques were used for the determination of different types of lipid.

In order to further characterize the relationship of the protein with the lipid, a quantitative estimation of the different types of amino acid present in the peptide portion of the molecule were determined. This was done by means of ion-exchange column chromatography.

REVIEW OF LITERATURE

The serum lipoproteins were of great importance in lipid transport and metabolism (23), and the circulating lipoproteins are the best evidence of the specific role of proteins in the transport of non-protein substances. Lipids, being insoluble in water, are transported by means of this physical union with proteins. These molecular associations were discovered by Macheboeuf (24) in 1928, and were called cenapses.

Lipids are linked to certain specific proteins immunologically different from other plasma proteins, and these proteins are not found in the free state when the lipids are absent. These associations are of quite definite structure and composition, and do not appear to be the result of random association of the lipid and protein (36).

The chemistry of both the protein and lipid moieties of the serum lipoproteins has been actively investigated because of the interest in their relationship to disease. Some investigations have been carried out on rat, pig, rabbit, dog, and chicken serum lipoproteins. However, most of the investigation and development of procedures has been on human serum lipoproteins. A satisfactory procedure for the isolation and physical characterization of human serum lipoproteins has been devised in the ultracentrifugal flotation method of Gofman and his associates (14). This method has been adopted and is widely used as a standard procedure.

As in the case of human lipoproteins, the most complete and definitive resolution of serum lipoproteins of the chicken has been achieved by the use of ultracentrifugal techniques (41). Several authors have determined the flotation constants of serum lipoproteins of the adult chicken and the chick (19,22,42,43). The methods employed have generally approximated those

developed by De Lalla and Gofman (14) for ultracentrifugal analysis of serum lipoproteins of the human.

The main fractions obtained by ultracentrifugal separation of normal human serum lipoproteins form distinct classes (50). On the high-density side of 1.063 are the alpha-lipoproteins (HDL_1, HDL_2 and HDL_3); on the low-density side are the beta-lipoproteins (LDL_1 and LDL_2) and the Very-Low-Density(VLD) lipoproteins. On the extreme low density side are the chylomicra. These particles are listed in order of decreasing density, increasing lipid content, and diameter. The latter ranges from about 100 angstroms (HDL_3) to about 15,000 angstroms (the largest chylomicron).

The denser alpha-lipoproteins have been found to have the higher percentage of protein (52%); the lipids found in association with this protein were composed mainly of phospholipids (20-25%), cholesterol esters (10-15%) and small quantities of triglycerides and cholesterol. The amino acid composition differed slightly from the composition of other plasma proteins by having a lower amount of sulfur containing amino acids. The N-terminal amino acid was aspartic acid. From the immunological point of view, the protein showed some heterogeneity which has not been clearly interpreted (36).

The protein is necessary for the solubility of the lipids in water. However, the complete protein is not necessary, and Ayrault-Jarrier (5) showed that although 2/3 of the protein may be lost by partial proteolysis, the residual "lipoprotein" was still quite soluble.

The beta-lipoproteins are much more heterogeneous than the alpha-lipoproteins. By ultracentrifugation, groups of beta-lipoproteins were differentiated according to their flotation constant (46). The denser beta-lipoproteins ($S_{f1.063}$ approximately 6) were found to have a relatively constant composition, of 20% protein, 20% phospholipid, 35-40% cholesterol

esters, 10% free cholesterol and 5-10% triglycerides. The protein moiety had a molecular weight of 200,000 and its N-terminal amino acid was glutamic acid (36). The analysis of the protein moiety of the beta-lipoprotein showed nothing which could explain the specificity of its association with lipids. The low percentage of sulfur containing amino acids (as in alpha-lipoproteins) appeared to be the only distinguishing feature (46).

The very-low-density (VLD) lipoproteins ($S_{f1.063}$ from 20-400) were shown to be lower in protein (6-12%), richer in triglycerides (50-70%), and the cholesterol/phospholipid ratio was higher (about 1 to 1.2). The protein moiety, which was immunologically related to the protein of the $S_{f1.063}$ 0-20 beta-lipoproteins, appeared to include other antigenic sites, and analysis of N-terminal amino acids (threonine, serine, aspartic and glutamic acids) (37) suggested that several proteins or polypeptides were present. Gustafson separated three groups ($S_{f1.063}$ 20-50, 50-100, 100-400) by ultracentrifugation (16).

Since chylomicra contain very little protein (.5-1%) and more than 80% triglyceride, they may be excluded from the lipoprotein category. It is difficult, however, to establish criteria that will indicate the definite boundaries between chylomicra, lipomicra, and the very-low-density lipoproteins. The mode of linkage between the protein moiety and lipids, and the role of the protein in transport are unknown. The presence of the same protein as in alpha-lipoproteins leads the investigators to the hypothesis that the alpha-lipoproteins may have a part in the formation of chylomicra (38).

The first step in a study of the structure and linkage of lipoproteins is solvent extraction. Polar solvent mixtures such as those containing ethanol or methanol have been found to be far more effective than non-polar solvents alone in removing lipids from all serum lipoproteins (3). Their

effectiveness is due to the alcohol first removing the water molecules and weakening hydrogen bonds which are essential to the structural integrity of the lipoprotein. Once this structure has been impaired, non-polar solvents can remove the lipids readily. Various workers (4,39,40,47) have determined, by partial delipidization of their phospholipid-protein residues, that a single, distinct "apolipoprotein" was present in each of the two major lipoprotein classes. Gustafson(17) showed by partial delipidization and characterization of the phospholipid-protein residues of very-low-density (VLD) lipoproteins ($S_{f1.063}$ 20) that these lipoproteins were not homogeneous but contained three different protein-phospholipid complexes. On the basis of electrophoretic, immunochemical, and ultracentrifugal data, he reported that one was similar to apolipoprotein A, found with the alpha-lipoproteins, and one was similar to apolipoprotein B, previously found with the low-density beta-lipoprotein. He also indicated a new one which he called C. This C component seemed to have an exceptional binding capacity for phospholipids. It may be that because of this high affinity for phospholipids, even small amounts of apolipoprotein C play an important role in maintaining the structural stability of very-low-density lipoprotein microemulsions.

In order to determine something about the protein portion of the molecule, amino acid analyses were done (50). The data indicate that these proteins are not very different from other serum proteins but some differences occur between the high density and the low density lipoproteins. Margolis and Langdon (26) have carried out a careful study of the amino acids of low density beta-lipoprotein. The amino acid composition was compared with a number of other proteins. In contrast to structural lipoproteins, which have been reported to have an unusually high content of non-polar amino acid residues, the amino acid composition of beta-lipoprotein more closely resembled

that of soluble simple proteins. This might indicate that a larger contribution to the lipid-binding properties of the polypeptide may be made by electrostatic interactions than by hydrophobic bonding.

N-terminal amino acid analysis disclosed dissimilarities between the two groups of lipoproteins, and on this point the results of several groups of workers (4,37,45) were in complete agreement. By this method, it has been demonstrated that the protein moiety of beta-lipoproteins is quite distinct from that of the alpha-lipoproteins.

The mode of binding between lipids and the protein is still the concern of biochemists (36). The proteolytic destruction of beta-lipoproteins is difficult and proteases split off only a small peptide fraction. Apparently lipids protect the protein against proteases. The partial proteolysis allows the ether to better extract the lipids and indicates the protective role of the protein. Triglycerides, cholesterol esters, and cholesterol are easily extractable, either by agitation with cold ether or by addition of anionic and cationic detergents and extraction by ether without agitation. These neutral lipids are apparently simply dissolved in the lipid phase of the lipoprotein, i.e. bound to phospholipids by hydrophobic chains. The phospholipids are, on the contrary, bound by several linkages; in addition to the linkages between hydrophobic chains of proteins and phospholipids, there are salt linkages between opposite poles of protein and phospholipids. Half of the phospholipids may be removed in the same manner as the neutral lipids, as if the phospholipids were in a double leaf of which one face only would be bound to protein.

The elucidation of structure in a complex association such as lipoproteins requires as much information as possible regarding composition, size, and properties. Vandenheuval (50) has compiled data from many different

sources and has presented a structure for the low-density beta-lipoproteins. This model consists of a central core of water surrounded by a neutral lipid layer of triglyceride, cholesterol, and cholesterol esters. Surrounding the entire structure is a thin shell of phospholipid and hydrated protein. The model assumes the shape of a sphere whose diameter is 135 angstroms and whose neutral lipid layer is 22 angstroms thick, with the phospholipid-protein layer being 7 angstroms. Margolis and Langdon (27,28), through chemical and enzymatic modifications of the beta-lipoproteins, have proposed several other possible structures for the lipoprotein. These include models where a lipid core is completely surrounded by a thin layer of the protein, one where the peptide portion is partly hidden by the lipid core, and models similar to that presented by Vandenheuval. The model they consider most likely is one which has several smaller entities consisting of a lipid core surrounded by a thin layer of protein; these smaller complexes are then aggregated together.

Under normal conditions, non-laying pullets and roosters appear to lack some of the lipoproteins observed in the human (41). Using electrophoresis, several authors (10,18,30) have noted marked electrophoretic differences between the serum patterns of the male and female chickens, which were not found in any other species examined. Hens, known to be ovulating were observed to have very prominent peaks migrating just ahead of the gamma globulin region and also displayed prealbumin components, both of which were absent in roosters. These, as well as a great quantity of lipoid substance, could both be removed by extractions with ether at -40°C.

Clegg et.al. (9) showed that when a diethylstilbestrol solution was injected into cockerels, it brought about changes in the serum of these birds which were similar to the changes that occur when hens begin egg production. Scheide (41) also demonstrated the difference in the serum of control and estrogenized roosters by means of the ultracentrifuge.

Various studies (15,51) on the source of phosphorous found in the egg have shown that extra phosphorous appeared in the blood of birds during egg production. Many studies have been conducted concerning the association of phospholipid with the protein of lipoproteins. These were mainly extraction experiments with organic solvents, and were mentioned previously.

Clegg and Hein (10) and Clegg et.al. (11) used moving boundary electrophoresis and radioactive tracer techniques to determine the P^{32} activity associated with the blood serum and egg proteins of the chicken after the administration of radioactive phosphorous. They noted that electrophoretic components one and five of diethylstilbestrol injected rooster blood serum exhibited high phosphorous activity. Similar results were obtained with the electrophoretic components of laying hen blood serum.

A great increase in the lipoproteins ($S_{f1.063}$ 15) extending into the chylomicron range was observed by Scheide (41). This was accompanied by a decrease in the original heavy lipoproteins, and the appearance of a new phosphoprotein and a new heavier lipoprotein. Infante and Polonovski (20) noted that in three week old pullets treated with estradiol cyclopentylpropionate, there was an increase in the slow moving beta fraction of lipoproteins and a marked decrease and practical disappearance of alpha-lipoproteins. A great increase in the P^{32} activity of the serum was also noted.

Evidence seems to indicate that either when the hen starts egg production or when the cockerel or young non-laying pullet is treated with diethylstilbestrol or some other related estrogenic compound, certain components increase in amount and some totally new ones appear. Accompanying these increases in certain serum components are striking decreases of most normal serum components.

Apparently, the chicken liver possesses a definitely limited economy

with respect to serum protein production, resulting in deficiencies of certain serum components when others are being produced by a "crash program" such as is initiated by the injection of estrogen (41).

Isolation of a high lipid and phosphorous containing protein fraction has been performed. Using salt-fractionation, ultracentrifugation and preparatory electrophoresis, Misra (29) has purified component 6 so that it was homogeneous to electrophoretic and ultracentrifugal analysis, although gradient ion exchange chromatography indicated a slight heterogeneity. Electrophoretic and ultracentrifugal analysis shows that component 6 was comparable to the very-low density or the low-density beta lipoproteins of human serum (29,41).

Malik showed by DNP amino terminal group analysis that there is a heterogeneity in this fraction. The amino terminal group analysis of Malik showed isoleucine and alanine before delipidization and isoleucine, alanine and threonine after delipidization. Lipoproteins of similar properties in the human exhibit serine, threonine, aspartic and glutamic acids as N-terminal amino acids. The fact that Malik found one less amino terminal group before delipidization suggests that the lipid may be masking the protein, as proposed by authors working on human serum lipoproteins (23).

In an attempt to further characterize the linkage between lipid and protein in component 6 of diethylstilbestrol treated birds, amino acid analysis, differential extraction with organic solvents, and lipid analysis by thin layer chromatography were performed.

MATERIALS AND METHODS

Blood Serum Sample

Serum was obtained from the blood of either White Rock or Leghorn cockerels. The cockerels were injected subcutaneously with 7.5 mg. of diethylstilbestrol per 0.5 ml. propylene glycol, daily for seven days. On the eighth day, the birds were sacrificed by severing the jugular vein and orotic artery and the blood was collected in 50 ml. centrifuge tubes. The blood was allowed to clot at 37°C for about four hours(7). It was then centrifuged at 3,000 revolutions per minute for 20 minutes at 4°C. The serum was decanted and stored overnight at 7°C.

Component 6

Initial isolation of component 6 was attained by means of the ultracentrifuge.[1] Cellulose nitrate tubes were filled with 11.3 ml. of serum and capped tightly with aluminum caps. The serum was centrifuged for 16 hours at 40,000 revolutions per minute at 0°C. At the end of this period of centrifugation, three major layers were observed. The top layer was a clear yellow substance with the appearance of set gelatin and the consistency of chilled cream. The bottom layer was hard, sticky, semi-solid and red in color. The middle layer was a pale yellow liquid. The top layer was removed very carefully by means of a spatula. This top fraction was found by Misra (29) to be essentially component number 6. After removal from the centrifuge tube, the sample was washed for several minutes with cold double distilled water. It was then dissolved in chilled 0.05 M borate sulfate buffer, pH 8.6, and

[1] Spinco Model L

dialyzed overnight against this buffer. Homogeneity of the fresh sample was examined by means of polyacrylamide disc electrophoresis (13,34). Both 7.5% and 3.75% gel concentrations were used. The buffer used was Tris-glycine buffer pH 8.2 - 8.4. Electrodes were placed in each reservoir and the polarity set so that the sample ions migrated toward the small-pore gel. Voltage was applied until the marker dye applied to the upper buffer reservoir was seen at the bottom of the tube (usually about half an hour). At the end of the running time, the gels were removed and placed in a solution of amido black dye, which fixed and stained the protein. Excess dye was removed from the gels by electrophoresis with 7.5% acetic acid solution or by washing with the acetic acid. The gels were then preserved in this solution.

Delipidization Procedures

Total lipids were determined by the method of Sperry and Brand (43), who have presented a method which permitted the direct determination of unmodified lipids. Into 8.3 ml. of pure methanol in a 25 ml. volumetric flask, one ml. of blood serum or solution of component 6 was pipetted, while swirling the flask. The solvent was brought almost to boiling on a steam bath. After cooling the flask to room temperature, chloroform was added to the mark and the contents were thoroughly mixed and filtered. Twenty ml. of the filtrate were pipetted into a 25 ml. glass stoppered cylinder, which had been checked previously for tightness of the stopper. Four ml. of water were added, the stopper inserted and the contents were shaken vigorously for 1 minute. The cylinder was allowed to stand overnight. The upper phase was clear; and the lower phase usually became clear, but sometimes it remained slightly cloudy. The upper phase was removed as completely as possible by the use of a water aspirator. Because the volume was small, the residue was

removed by washing. The wash was prepared previously for this purpose as follows: 96.3 ml. of chloroform/methanol 2:1 and 23.7 ml. of a solution containing 20 mg. of $CaCl_2$ per 100 ml. of water were thoroughly equilibrated in a separatory funnel and allowed to stand until the phases were separated and the upper phase was clear. The lower phase was discarded. The remaining solution had approximately the same solvent concentration as the upper phase obtained in the purification of the lipid extracts. Three ml. of this solution were added to the cylinder, taking care to wash the sides of the cylinder and avoid mixing of the lower phase. The cylinder was then rotated back and forth in a vertical position to mix the residue of the original upper phase with the wash solution, which was removed as before. The washing procedure was repeated two more times. Two ml. of methanol were added to the cylinder, the contents mixed, and the clear solution was transferred by means of a filtering stick to a 50 ml. erlenmeyer flask, which has been previously rinsed with 2:1 chloroform/methanol, dried to constant weight in a dessicator and weighed. The solution was evaporated to dryness at 35-40°C in a stream of nitrogen. These samples were then dried to constant weight in an evacuated dessicator overnight and weighed. One flask was kept as a control in the dessicator, and the weights of the other flasks were corrected for any change in their weight. This correction was usually small, but it might be appreciable if there was a marked change in atmospheric conditions.

Partial extraction (3) was carried out at 4°C. in a 100 ml., glass stoppered, round bottomed flask. Five ml. of lipoprotein solution and 40 ml. of peroxide-free ether were added to the vessel. The flask was flushed with nitrogen, and sealed. The flask was revolved at a speed of 20-23 revolutions per minute, and extraction continued for 16 hours. The phases were then separated by centrifugation. The lower phase was washed once with ether and the

wash was combined with the upper phase. The lipid solution was evaporated to dryness in a stream of nitrogen and dissolved in 10 ml. of a 2:1 chloroform/methanol mixture. This solution was stored under nitrogen at 4°C.

Total extraction of lipid material from component 6 was achieved by using a 2:1 chloroform/methanol mixture. This was carried out at 4°C in a 100 ml. glass stoppered round bottomed flask. Five ml. of lipoprotein solution and 40 ml. of chloroform/methanol were added to the flask. The flask was flushed with nitrogen, sealed, and revolved about its horizontal axis for 16 hours at 4°C. The phases were then separated and the upper phase was washed with the chloroform/methanol mixture. The lipid solution was evaporated to dryness in a stream of nitrogen, at 4°C, and redissolved in 10 ml. of chloroform/methanol, and stored under nitrogen at 4°C until used.

These two extractions produced the lipid solutions used in thin layer analysis of the lipid portion of component 6.

In order to prepare large quantities of delipidized sample for hydrolysis and chemical analysis, the method of Sperry and Brand (48) was used. Eighty ml. of methanol were placed in a 250 ml. flask and 9.5 to 10 ml. of dialyzed component 6 were added dropwise with swirling to the methanol. Eighty ml. of chloroform were added and the mixture was heated to almost boiling (approximately 55°C). No stoppers were used in the flasks. The mixture was then cooled to room temperature. A clear yellow solution with white fluff floating in it was produced. This was the delipidized protein. When cooled to room temperature, the flasks were brought to 250 ml. volume with chloroform, well mixed, and allowed to stand for two hours at room temperature. The protein then settled to the bottom and a large portion of the chloroform/methanol was carefully drawn off with a water aspirator. The portion of solvent which remained was removed by filtration using a glass sintered funnel, and the pro-

tein precipitate was washed with about 50-100 ml. of 2:1 chloroform/methanol and pressed dry. This cake was dried in an oven at 105°C for ½ hour, air equilibrated and weighed.

Lipid Analysis by Thin Layer Chromatography

The apparatus used in this thin layer analysis was developed by Egon Stahl (49). The plates were run to 15 cm. on every run and then removed (2). Standard mixtures obtained from the Applied Science Laboratories were run along with the unknown mixtures of lipids extracted from component 6. These standards were as follows:

1. Cholesterol oleate, methyl oleate, triolein, and oleic acid. The solvent system used was petroleum ether/ether/acetic acid, 90/10/1 by volume.
2. Cholesterol, cerebrosides, sulfatides, and sphingomyelin. The solvent system used was chloroform/methanol/water, 90/10/1 by volume.
3. Cholesterol, phosphatidyl ethanolamine, lecithin and lysolecithin. The solvent system used was chloroform/methanol/water, 60/25/4 by volume.
4. Monogalactosyl diglyceride, three cerebrosides, and lecithin. The solvent system used was chloroform/methanol/acetic acid, 100/25/4 by volume.
5. Palmitic acid, lecithin, lysolecithin, and alpha-glyceryl phosphate. The solvent system used was chloroform/methanol/water, 65/25/4 by volume.

The plates were divided into channels 1½ inches wide and one spot was placed in each channel. Standard mixtures were spotted along with the various lipid extraction samples described previously. The plates were then developed in the solvent system appropriate for the standard. After development in the four different solvent systems relevant to each standard mixture, the lipid spots were made visible either by placing the plates in a tank saturated with iodine vapors, or by charring the spots by spraying with sulfuric acid and

potassium dichromate and heating in an oven at 150°C until the spots were completely charred. Both of these methods revealed the same number of spots.

To determine the quantity of cholesterol in the total sample, the Cholestex reagent for serum cholesterol was used.[1] Phospholipids were determined by the wet digestion method of Youngburg and Youngberg (35), after extraction of lipids from component 6 by the method of Sperry and Brand, as previously described. An aliquot was taken from the same extraction from which total lipids were determined, and was used for the determination of phospholipids.

In order to approximate the amounts of lipids in component 6, several comparisons were made. Thin layer chromatography showed the presence of four general classes of lipids; triglycerides, phospholipids, cholesterol esters, and cholesterol. Total lipids, phospholipids, and cholesterol were determined by the methods previously described. In order to estimate the amounts of cholesterol esters and triglycerides, the charred thin layer plates were examined by means of a densitometer and the sizes of the peaks of the phospholipids, whose concentration had been previously determined. Since all samples spotted were of the same concentration, this gave an approximation of the amounts of each different class of lipid present. Not all the concentrations could be estimated by means of thin layer chromatograms and the densitometer, because the triglycerides ran with the cholesterol when using the chloroform/methanol/water 65/25/4 and 90/10/1 solvents.

A moisture determination was carried out on component 6. Bottles were weighed to constant weight by heating to 110°C and cooling in an evacuated dessicator. Samples of native component 6 were weighed and placed into a

[1] Omni-Tech Inc.

vacuum oven at 110°C for 2 hours. Samples were removed and cooled in an evacuated dessicator. Samples were weighed to constant weight and the per cent moisture calculated.

The quantity of protein in both the native and delipidized component 6 was estimated by means of the microkjeldahl procedure (33) and the protein estimated from the N_2 per cent. The sample was weighed by means of a charging tube and transferred into the microkjeldahl flask, where it was digested with concentrated sulfuric acid in the presence of a catalyst until the mixture became colorless. This digestion was aided by the addition of hydrogen peroxide. The flasks were cooled and ammonia determined by distilling the mixture after the addition of sodium hydroxide. The ammonia was dissolved in boric acid solution and titrated directly with standardized sulfuric acid using a methyl-red/methylene-blue indicator. In order to estimate the amount of protein in the undelipidized sample, the amount of nitrogen contained in the phospholipids was determined and subtracted from the per cent of total nitrogen as determined by the microkjeldahl.

To determine the amount of nitrogen in component 6 contributed by amides, a sample of delipidized component 6 was weighed and placed in a hydrolysis tube (1,44). One ml. of 2N. HCl, freshly prepared with deionized water, was added for every mg. of protein. The tubes were frozen in a dry ice and ethanol mixture, evacuated with a vacuum oil pump, and sealed while in a vacuum with a propane torch. The hydrolysis was carried out in a chamber kept at a constant temperature of 110° by refluxing toluene. The samples were hydrolyzed for 1, 2, and 3 hours. At the end of the hydrolysis period, the tubes were cooled in ice. After filtering the hydrolysate, a two ml. aliquot was taken and diluted to 10 ml. One ml. of this dilution was placed

in a test tube, and 1 ml. of 0.1 N NaOH, 1 ml. of 25% Na Phenolate, 1 ml. of 0.06 Na Hypochlorite, and 1 ml. of 0.005% Na Nitroprusside were added simultaneously. This last reagent was stored in a .5% stock solution at 7°C and diluted 1/100 just prior to use. The pH of the final mixture of reagents and hydrolysate must be 11.2 ± 0.2. The normality of the NaOH reagent was adjusted to bring the final mixture to this pH. The color was allowed to develop for 1 hour, diluted with 5 ml. of deionized water, and read on the Beckman DB at a wavelength of 625 millimicrons. The sample size was adjusted such that approximately 0.1 to 0.15 micromoles of ammonia were contained in each test tube. Every reagent and dilution of sample was prepared with deionized water to minimize the amount of extraneous ammonia. A blank was prepared with all reagents except protein. A standard curve of ammonium sulfate was plotted with the absorbance along the abcissa and the micromoles of ammonia along the ordinate. To determine the micromoles of ammonia contained in each sample, the absorbance was read off the standard curve shown in Appendix A. This value was then divided by the sample size in each tube to obtain the micromoles of ammonia per mg. of sample.

Amino Acid Analysis

Hydrolysis of component 6 was carried out in an evacuated container (12). A ten to fifteen mg. sample of the delipidized, air equilibrated sample was weighed by means of a charging tube and placed into a 15 ml. capacity hydrolysis tube. These tubes had been previously cleaned with chromic acid, then rinsed with double distilled water and finally with 1N HCl. The residual HCl was removed in an air oven at 100°C, and the tubes were stored inverted in a covered container to prevent deposition of NH_4Cl from the air.

The protein in the tube was suspended in 1 ml. of 6 N HCl for every five

mg. of sample. The contents of the tube were then frozen in a dry ice/ethanol mixture, evacuated with an oil pump for 10 to 15 minutes, and then sealed with a propane torch while the sample was still frozen and under a vacuum.

Hydrolysis was conducted at $110^{\circ} \pm 1^{\circ}$ for 20, 40, or 70 hours. The temperature was kept constant by placing the tube in a chamber heated by refluxing toluene.

After hydrolysis, the tube was cooled to room temperature, and stored at $4^{\circ}C$ or in the deep freeze until use. To remove the sample from the hydrolysis tube and prepare it for amino acid analysis, the narrow neck of the tube was scored with a sharp file and snapped off. The contents of the tube were transferred quantitatively with double distilled water to a 50 ml. round bottomed flask with a ground glass joint. This flask was then attached to the joint of a small rotary flash evaporator, and placed in a $40^{\circ}C$ water bath. Most of the HCl was removed in 20 minutes; and flask was left on the evaporator at $40^{\circ}C$ for 20 more minutes to ensure the complete removal of HCl. Before analysis, the residue was dissolved in 1 ml. of 0.2 M sodium phosphate buffer of pH 6.5. The nearly neutral solution (there is no loss of NH_3 at this pH) (12) was allowed to stand for four hours to permit air oxidation of any cysteine to cystine, and was then brought to about pH 2 by the addition of 0.6 ml. of freshly prepared 1 N HCl. The resulting solution was transferred quantitatively to a 10 ml. volumetric flask by the use of 1 ml. aliquots of pH 2.2 sodium citrate buffer containing 5 ml/l of thiodigylcol. A 1 ml. aliquote of this solution was used for each analysis. Chromatography was performed on Amberlite IR-120 ion exchange resin, and on Aminex A-4 and Aminex A-5.[1]

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[1] Bio-Rad Company.

In preparing and operating columns for the separation of acidic and neutral amino acids, two different buffers were employed (31). Two tenths normal sodium citrate buffer pH 3.25 was run first and then after the elution of glycine, the buffer was changed to a 0.2 N sodium citrate buffer of pH 4.25. For separation of the basic amino acids, a different column was used, and a different resin. The buffer used for this elution was a 0.35 N sodium citrate buffer of pH 5.28. The table below illustrates the preparation of these buffers.

TABLE I.

pH	Na Conc. N	Citric Acid H_2O G	NaOH 95% G	HCl Conc. Ml.	Final Vol. Liters	Phenol to final Vol. G	TG* Ml.
3.25	0.20	840	330	426	40	40	10
4.25	0.20	840	330	188	40	40	10
5.28	0.35	491	288	136	20	20	--

*TG is thiodiglycol

A solution of 0.2 N sodium hydroxide was employed to regenerate the columns.

Preparation of columns:

Three different types of resin were used to complete amino acid analysis of the delipidized portion of component 6 (8, 31). Aminex A-4 spherical resin was used to resolve the basic amino acids. The amino acids, serine and threonine, were not completely separated by the Aminex A-4 resin; therefore, Amberlite IR-120 resin was used for this purpose (31).

Approximately 40 grams of Aminex A-4 resin was placed in a 150 ml. beaker (8). Eighty ml. of pH 3.25 buffer was added and the resin was slurried. The resin was allowed to settle and the buffer was decanted so that a volume of

40 ml. remained. .Before pouring the resin, the column was filled with 3.25 buffer, and nitrogen was used to force the buffer through the teflon disc at the bottom of the column. The resin was reslurried in the buffer, and this very thick slurry was poured into the column in one continuous pour. The buffer line was attached and the pump was set to pump buffer at a rate of 60 ml/hr. The bed height was adjusted to 50 cm. ± 2 cm. and a teflon disc placed on top of the packed column. The initial back pressure for this column operating at 50°C, packed with Aminex A-4 resin, was usually no greater than 90 psi at buffer flow rate of 60 ml/hr.

The column used to analyze the basic amino acids was packed with Aminex A-5 spherical resin. Approximately 10 grams of resin were placed in a 50 ml. beaker, and 30 ml. of 5.28 buffer was added to form a slurry. The resin was then allowed to settle and the buffer decanted until a total volume of 15 ml. remained. The column was poured as stated for the long column. The column height was adjusted to 8 cm. The initial back pressure for the short column operating at 50°C., packed with Aminex A-5 resin, was usually no greater than 20 psi at buffer flow rate of 30 ml/hr.

The Amberlite IR-120 column was operated following the procedure of Moore and Stein (31). This column also separated the acidic and neutral amino acids. About 100 ml. of settled resin were needed for the column, which was 150 cm. long, and a diameter of .9 cm. The average size of these particles of resin was about 56 microns. The commercially prepared resin was ready to slurry with buffer pH 4.25. The resin was rinsed several times to remove the fine particles, which cause the column to pack too tightly, and the resin was slurried in several hundred ml. of buffer. The column was prepared in five or six sections. Before the first section was poured, the outlet tube was closed. As soon as the slurry of resin was poured, the outlet tube was opened,

and about 2 cm. of resin bed was allowed to form under gravity flow. Pressure was then applied to the column to pack it. The pouring was done at room temperature. After the resin had settled to constant height all of the supernatant buffer was withdrawn by suction and the next section was poured and packed, a funnel was attached on the top of the column to allow enough buffer and resin to be added for one section. The final height of the resin was several cm. greater than 150 cm. to allow for further packing during operation. This column was operated at a flow rate of 12 ml/hr. at 50°C with a back pressure of approximately 20 psi.

Before use, the 150 cm. column was washed with .2 N sodium hydroxide and equilibrated with boiled buffer at pH 3.25. This procedure was also used after each amino acid analysis run to clean the column of the residual basic amino acids. The Aminex A-4 column which separated the acidic and neutral amino acids was also regenerated by this method after every run.

The optimum load for all the columns (Aminex A-4, Aminex A-5, and Amberlite) was 0.5 to 1.0 micromoles of amino acid or 1 to 2 mg. of hydrolyzed protein (29). The initial buffer for the acidic columns (Aminex A-4 and Amberlite) was pH 3.25, the columns were maintained at 50°C throughout the analysis, and the effluent was collected in 1 ml. fractions, which were analyzed photometrically by the addition of a modified ninhydrin reagent (32). An eluent at pH 4.25 was introduced at a time designed to allow valine to emerge with the new buffer. The change was made at the lowest point between the glycine and alanine peaks. For the Aminex A-4 column, this change occurred at an effluent volume of 80 ml. and at a time of approximately 80 minutes. For the Amberlite column this change would occur at an effluent volume of 173 ml. and at a time of approximately 14.5 hours. A run for the Aminex A-4 column was complete in approximately 3 hours, and a run in the Amberlite column was complete in approximately 27.5 hours.

Samples analyzed in the basic column of Aminex A-5 resin required only one buffer, pH 5.28, and the run was completed in a little less than 4 hours with a total effluent volume of 115 ml. This column was maintained also at a constant temperature of 50°C throughout operation. On completion of an analysis of the acidic and neutral amino acids in the Aminex-4 and Amberlite columns they were regenerated to remove all the residual basic amino acids. No regeneration, however, was needed for the Aminex A-5 column, which separates the basic amino acids, because the pH of the effluent buffer was high enough to clear the column of all acidic amino acids before the basic amino acids were separated. Most samples did not contain any ninhydrin-positive constituents with a greater retardation than arginine.

In order to apply a sample to the column, all of the buffer must first be removed from the top of the column above the pellet. A sample of 0.5 to 1.0 micromoles of amino acid or 1 to 2 mg. of protein contained in 0.5 to 1.0 ml. of pH 2.2 sodium citrate buffer was placed into the column by means of a pipette. Care was taken to apply the sample directly to the top of the column. The sample was forced into the column by about 25-30 psi of nitrogen pressure. When the sample was on the column, the sides of the column were washed with pH 2.2 buffer and the buffer was forced into the column in the same manner. This washing procedure was repeated twice more. After the last wash was in the column, the space above the resin was filled with the buffer, and the pump started. Fractions of 1 to 1.5 ml. were collected in a fraction collector.

The reagent used to develop the fractions of amino acids eluted from the columns was a modified ninhydrin reagent developed by Moore and Stein (32). This reagent was prepared by dissolving 10 grams of ninhydrin and 1.5 grams of hydrindantin in 375 ml. of methyl cellosolve.[1] This mixture was stirred

[1] Peroxide free

carefully so as not to incorporate air bubbles. After the hydrindantin and ninhydrin were dissolved, 125 ml. of 4 N sodium acetate buffer pH 5.5 were added. This buffer is concentrated enough to take care of the pH differences in the samples analyzed. The reagent was transferred to a dark glass bottle, and stored under nitrogen at a temperature of 4°C in the dark. When stored under these conditions, the reagent was stable for about 5 to 7 days with reproducible results.

To analyze the samples, one half ml. of ninhydrin reagent was added to each 1 ml. sample. These tubes were then shaken to ensure homogeneity and heated in a boiling water bath for 15 minutes. They were then removed and cooled to room temperature. The samples were diluted with 50% ethanol before analysis. Tubes were read on a Bausch and Lomb spectronic-20 at a wavelength of 570 millimicrons. Tubes analyzed for proline and cystine were read at 440 millimicrons also.

A standard curve was made using graduated concentrations of leucine as the reference amino acid. (See Fig., Appendix B.) The absorbance was plotted on the abcissa and the micromoles of leucine along the ordinant. Moore and Stein (32) have related the color yield of all the other amino acids and ammonia to leucine (see table below), using the modified ninhydrin reagent previously described.

TABLE II.

Aspartic acid	.94	Methionine	1.02
Threonine	.94	Isoleucine	1.02
Serine	.95	Leucine	1.00
Glutamic acid	.99	Tyrosine	1.00
Proline	.225	Phenylalanine	1.00
Glycine	.95	Lysine	1.10
Cystine	.55	Ammonia	.97
Valine	.97	Arginine	1.01

These values were checked by running a standard Beckman Mixture (6) of all the amino acids. They were in very close agreement for both types of resin. A graph and table for the standard runs for the columns are shown in Appencix C.

Concentrations for the amino acids were calculated by averaging the absorbances of one peak and reading this number off the standard leucine curve to obtain the micromoles of amino acid. This value was then corrected by dividing by the color yield shown in Table II. To obtain the micromoles of amino acid per mg. of sample, this value was divided by the mg. of sample applied to the column.

Amino acid composition may be expressed as micromoles per mg. or as moles per 10^5g of sample. It may also be expressed as a percentage of the total micromoles of amino acid, or the values may all be normalized to aspartic acid. In the present discussion of amino acid concentration in component 6, it is calculated both as micromoles per mg. and as moles per 10^5g of hydrolyzed delipidized component 6.

Since Tryptophan is destroyed during acid hydrolysis, a separate determination was made to determine the concentration of this amino acid in component 6 (21). To 1 ml. of concentrated sulfuric acid, 0.5 ml. of alcoholic sodium hydroxide was added slowly. To this mixture, either the protein sample or the solution of tryptophan used to prepare the standard curve was added. Next 5 ml. of glacial acetic acid was introduced with continuous stirring, and then 2 drops of potassium persulfate reagent were added. The final volume was approximately 7 ml. For free tryptophan, the color developed to a maximum in approximately 10 min., decreasing fairly rapidly after 20 min. The optical density was measured at 550 millimicrons between 10 and 20 minutes after adding the persulfate. This value was read from the standard curve included in the appendix to obtain the concentration of tryptophan in the sample.

The phosphorous of delipidized component 6 was determined by the method of Fiske and Subbarow (35).

RESULTS AND DISCUSSION

Polyacrylamide disc electrophoresis of component 6 revealed only one major peak which moved a very short distance into the running gel. It is not possible to state whether or not component 6 is pure from this data; however, Misra (29) by means of starch gel electrophoresis, free electrophoresis, and ultracentrifugal analysis found essentially one entity involved in component 6 also. Plate I shows a comparison of the total serum of the diethylstilbestrol injected bird, and ultracentrifugally prepared component 6.

In order to study the lipid composition of component 6, thin layer chromatograms of standard lipid mixtures, an ether extraction and chloroform/methanol extracts of samples which had first been extracted with ether, were chromatographed. These chromatograms were developed by staining with iodine vapors or by charring with-sulfuric acid and potassium dichromate. Photographs of these charred plates are shown in Plates II, III, IV and V. The standard spots were identified by comparing them with standards furnished by Applied Science Laboratories. Since the same solvents were used to run the chromatograms as were used by the company in preparing sample chromatograms, the distribution of lipid components in the standard runs matched the samples furnished by the company.

In the extract from the totally delipidized component 6, a large amount of triglycerides, smaller amounts of phosphatidyl ethanolamine and lecithin, and a very small amount of cholesterol ester were observed. This is illustrated in T of Plates II and III. The first impression from these chromatograms was that a large amount of cholesterol was present; however, it was later observed that in the chloroform/methanol/water solvent system, the triglycerides moved with the cholesterol. Therefore, a separate determination was made to determine the cholesterol present in the lipid fraction of component 6.

EXPLANATION OF PLATE I

Plate I shows typical electrophoretic patterns of the serum of diethylstilbestrol injected birds, and ultracentrifugally prepared component 6 in 7.5% polyacrylamide gel. Picture A shows component 6. Picture B shows the total serum. The dark band at the top of the running gel of picture A is component 6.

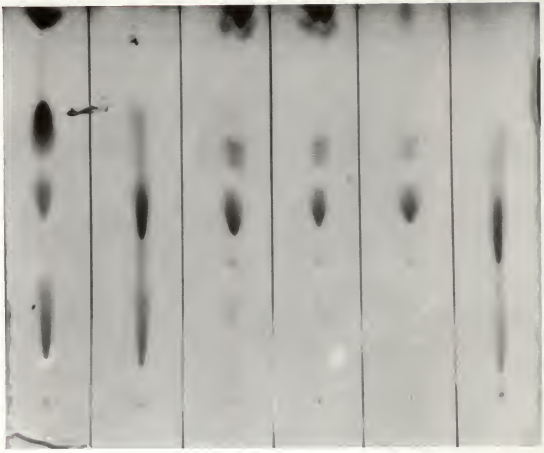
A B

EXPLANATION OF PLATE II

One dimensional ascending chromatograms of standard lipid mixtures number three and five were run. Number three contains cholesterol, phosphatidyl ethanolamine, lecithin, and lysolecithin. The standard mixture number five contains palmitic acid, lecithin, lysolecithin, and alpha-glyceryl phosphate. A five lambda aliquot of each of the two standard solutions was used for spotting the chromatogram. A 10 lambda aliquot of the chloroform/methanol extract of component 6, and a 10 lambda aliquot of the ether extract of component 6 were used. A 25 lambda aliquot was used to spot the chloroform/methanol extract of the ether extracted component 6.

Only one solvent system was used to resolve the lipids shown in the photograph. Plates were developed in chloroform/methanol/water, 60/25/4 by volume. The time for running was approximately 25 minutes, and the plates were run to 15 cm. and removed from the solvent. The spots were made visible by charring with sulfuric acid and potassium dichromate and heating in 150°C oven.

Spots listed from top to bottom for standard mixture number three are: First, cholesterol followed by phosphatidyl ethanolamine, lecithin, and lysolecithin. For standard number five, the farthest spot from the origin is palmitic acid, followed by lecithin, lysolecithin, and next the origin, alpha-glyceryl phosphate. In the chloroform/methanol extract of component 6 and in the cold ether extract of component 6, at the top was a mixture of triglyceride and cholesterol (spot A), next was phosphatidyl ethanolamine (spot B), followed by lecithin (spot C). In the chloroform/methanol extract of the ether extracted component 6, the first spot was cholesterol, the second spot from the top was identified as phosphatidyl ehtanolamine and following that was lecithin.



3 5 T P T•P 5

Key:

3 . . . . . Standard Mixture number 3

5 . . . . . Standard Mixture number 5

T . . . . . Chloroform/methanol 2:1 extract of component 6.

P . . . . . Cold ether extract of component 6.

T•P . . . . . Chloroform/methanol extract of ether extracted component 6.

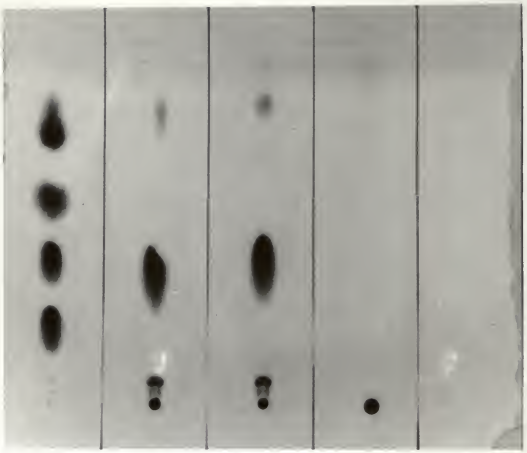
Plate was run 15 cm. up from the point of application.

EXPLANATION OF PLATE III

One dimensional chromatograms of standard lipid mixture number 1 were run. Number 1 contained cholesterol oleate, triolein, and oleic acid. A one lambda aliquot of the standard mixture number 1 was used for spotting the chromatogram. A 10 lambda aliquot of the chloroform/methanol extract of component 6, and a 10 lambda aliquot of the ether extract of component 6 were used. A 25 lambda aliquot was used to spot the chloroform/methanol extract of the ether extracted component 6.

Only one solvent system was used to resolve the lipids shown in the photograph. Plates were developed in petroleum ether/ether/acetic acid, 90/10/1 by volume. The time for running was approximately 45 minutes, and plates were run to 15 cm. and removed from the solvent. The spots were made visible by charring with sulfuric acid and potassium dichromate and heating in a 150°C oven.

Spots listed from top to bottom for standard mixture number 1 were cholesterol oleate, followed by methyl oleate, triolein, and oleic acid. In the chloroform/methanol extract of component 6 and in the cold ether extract of component 6, at the top was a small amount of cholesterol ester (spot A), followed by a large amount of triglyceride (spot B). Spots near the origin are not identifiable with this standard. In the chloroform/methanol extract of the ether extracted component 6, no triglyceride or cholesterol ester was observed.



Key:

1 . . . . . Standard Mixture number 1

T . . . . . Chloroform/methanol 2:1 extract of component 6.

P . . . . . Cold ether extract of component 6.

T•P . . . . . Chloroform/methanol extract of ether extracted component 6.

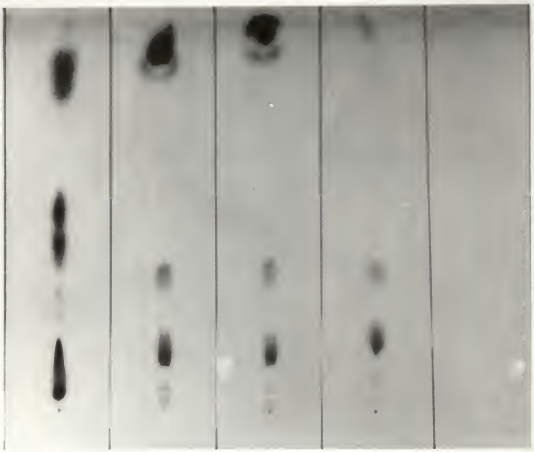
Plate was run 15 cm. up from the point of application.

EXPLANATION OF PLATE IV

One dimensional chromatograms of standard lipid mixture number 2 were run. Number 2 contained cholesterol, cerebrosides, sulfatides, and sphingomyelin. A five lambda aliquot of the standard mixture number 2 was used for spotting the chromatogram. A 10 lambda aliquot of the chloroform/methanol extract of component 6, and a 10 lambda aliquot of the ether extract of component 6 were used. A 25 lambda aliquot was used to spot the chloroform/methanol extract of the ether extracted component 6.

Only one solvent system was used to resolve the lipids shown in the photograph. Plates were developed in chloroform/methanol/mater, 90/10/1 by volume. The time for running was approximately 45 minutes, plates were run to 15 cm. and removed from the solvent. Spots were made visible by charring with sulfuric acid and potassium dichromate and heating in a 150°C oven.

Spots listed from top to bottom for standard mixture number 2 were cholesterol, followed by cerebrosides, a little below center. Next are sulfatides, which did not separate; and very near the origin, is sphingomyelin. None of the spots in component 6 were identifiable with this standard.



Key:

2 . . . . . Standard Mixture number 2

T . . . . . Chloroform/methanol 2:1 extract of component 6.

P . . . . . Cold ether extract of component 6.

T•P . . . . . Chloroform/methanol extract of ether extracted component 6.

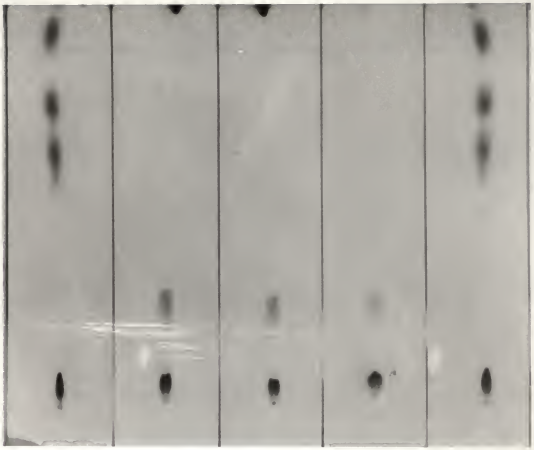
Plate was run 15 cm. up from the point of application.

EXPLANATION OF PLATE V

One dimensional chromatograms of standard lipid mixture number 4 were run. Number 4 contained monogalactosyldiglyceride, three cerebrosides, and lecithin. A 5 limbda aliquot of the standard mixture number 4 was used for spotting the chromatogram. A 10 lambda aliquot of the chloroform/methanol extract of component 6, and a 10 lambda aliquot of the ether extract of component 6 were used. A 25 lambda aliquot was used to spot the chloroform/methanol extract of the ether extracted component 6.

Only one solvent system was used to resolve the lipids shown in the photograph. Plates were developed in chloroform/methanol/acetic acid, 100/25/4 by volume. The time for running was approximately 45 minutes, plates were run to 15 cm. and removed from the solvent. Spots were made visible by charring with sulfuric acid and potassium dichromate and heating in a 150°C oven.

Spots listed from top to bottom for standard mixture 4 are monogalactosyl diglyceride, followed by three cerebrosides, and close to the origin is lecithin. The only spot identifiable in component 6 extracts with this standard was lecithin, near the origin.



4 T P T·P 4

Key:

4 . . . . . Standard Mixture number 4

T . . . . . Chloroform/methanol 2:1 extract of component 6.

P . . . . . Cold ether extract of component 6.

T·P . . . . . Chloroform/methanol extract of the ether extracted component 6.

Plate was run 15 cm. up from the point of application.

The quantity of each of the lipids (triglyceride, phospholipid, cholesterol ester, and cholesterol) was estimated. Results of these determinations are shown in Table III. The quantity of total lipid in the native component 6 was determined by the method of Sperry and Brand (43). Component 6 was found to contain 60.9% lipid. The next step was the determination of total lipid phosphorous by the wet digestion method of Youngburg and Youngburg (35). The total lipid portion of component 6 was found to contain 0.302% phosphorous (see Table III). The amount of phosphorous was then multiplied by 25 to estimate the percent of phospholipid. The factor of 25 was based on lecithin, since most lipids appear to contain a much greater amount of lecithin than other phospholipid. The cholesterol determination indicated that 4.14% of the total lipid was cholesterol. To estimate the quantity of the other lipids present, the thin layer plates were passed through a densitometer. By comparing the relative sizes of the phospholipid peaks, the triglyceride peak, and the cholesterol ester peak, and knowing the concentration of phospholipid in the sample, it was possible to obtain an approximation of the relative amounts of the other lipids. Typical densitometer tracings of component 6 are shown in Plates VI and VII. According to the data in Table III, the lipid portion of component 6 contained 20% phospholipid, 4% cholesterol, 1% cholesterol ester, and 75% triglyceride. No free fatty acids were detected by the methods employed.

Phosphorous analyses, as described in previous sections, were run on the native sample, the totally delipidized sample, and on the lipid portion alone. The native sample contained 0.518% phosphorous and a very small amount, 1.28%, of this amount was still present in the protein portion after total delipidization (see Table III). Although this value was relatively small, it remained constant from sample to sample. This phosphorous not extracted by

TABLE III

	No. of Det'ns	Native Component 6	No. of Det'ns	Dry Component 6	No. of Det'ns	Lipid Portion	No. of Det'ns	(a) Delipidized Component 6	No. of Det'ns	(b) Ether Extr. Component 6
Total Lipid	3	60.9±0.9%	3	91.8±1.1%	--	100%	--	0.0%	--	--
% Phosphorous	5	0.581±0.015%	5	0.826±0.019%	3	0.802±0.02%	5	*0.137±0.002%	--	--
% Phospholipid	5	14.5±0.6%	5	28.5±0.8%	3	20.0±0.6%	--	--	3	**20 ± 5%
Cholesterol	5	2.52±0.05%	5	3.80±0.07%	5	4.14±0.08%	--	--	--	--
Triglyceride	3	43 ± 2%	3	65 ± 3%	3	75 ± 4%	--	--	3	0%
Cholesterol Ester	3	3.5±0.13%	3	1.3±0.2%	3	1.4±0.2%	--	--	--	--
% Moisture	3	32.7±1.4%	3	0.0%	--	--	--	--	--	--
% Nitrogen	6	1.19±0.02%	6	1.79±0.03%	--	--	5	11.3±0.02%	--	--
% Protein	6	5.41±0.09%	6	8.22±0.10%	--	--	5	70.7±1.2%	--	--
Amide Nitrogen	--	--	--		--	--	5	0.41±0.02%	--	--
% N due to Tryptophan	--	--	--		--	--	5	0.12±0.03%	--	--
% N recoverable from ion exchange	--	--	--		--	--	5	10.3±0.02%	--	--

*This value comprises 1.28±0.07% of the total phosphorous not extracted from the native component 6.

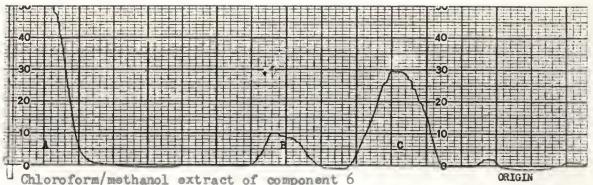
**This value is the percentage of phospholipid not extracted with the ether solvent. Twenty percent of the phospholipid was left behind.

(a) Component 6 extracted with chloroform/methanol 2:1

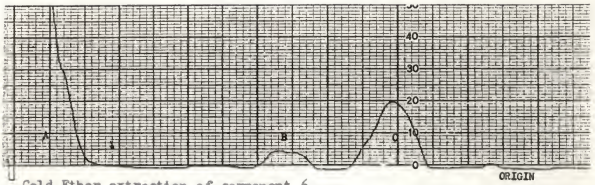
(b) Component 6 extracted with cold ether

EXPLANATION OF PLATE VI

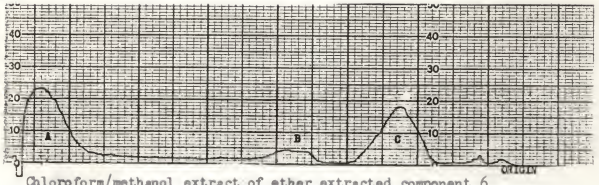
Plate VI is a typical densitometer tracing of Plate II, shown previously. Peak A (corresponding to spot A, Plate II) of the chloroform/methanol extract of component 6 and the ether extract of component 6 is comprised of triglyceride and cholesterol. Peak B is phosphatidyl ethanolamine, and peak C is lecithin (Peak B and C corresponding to spots B and C of Plate II). Peak A of the chloroform extraction of the ether extracted residue of component 6 is a small amount of cholesterol and a dark solvent front. Peak B and C are the same as stated above. By comparing the areas under the curves obtained for phospholipid, of the different extractions, it was found that about 20 ± 5% of the phospholipids were not extracted from component 6 by the cold ether, and were found when component 6 was reextracted with chloroform/methanol 2:1. This value is the average of three desnitometer tracings.



Chloroform/methanol extract of component 6
Sample size = 10 lambda Solvent: chloroform/methanol/water 60/25/4



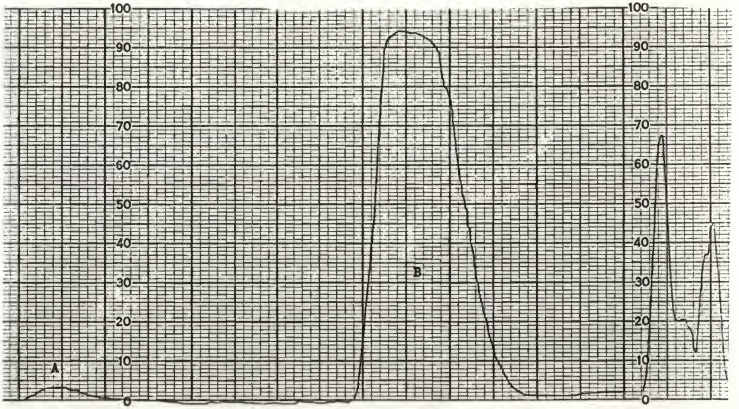
Cold Ether extraction of component 6
Sample size = 10 lambda Solvent: chloroform/methanol/water 60/25/4



Chloroform/methanol extract of ether extracted component 6
Sample size = 25 lambda Solvent: chloroform/methanol/water 60/25/4

EXPLANATION OF PLATE VII

Plate VII is a typrical densitometer tracing of chloroform/methanol extracts of component 6. This tracing corresponds to T in Plate III. Peak A (corresponding to spot A of Plate III) is cholesterol esters, peak B (corresponding to spot B of Plate III) is triglycerides. Component 6 was found to contain 75 $\pm$ 5% triglycerides, and approximately 1 $\pm$ 0.3% cholesterol ester by comparing the areas of the peaks above with the areas of the phospholipid peaks shown in Plate VI. These values were averages of three densitometric tracings.



Chloroform/methanol extract of component 6
Solvent: petroleum ether/ether/acetic acid
Sample size = 10 lambda

chloroform/methanol mixture may be the following: 1. a firmly bound phospholipid, 2. phosphorous bound to the protein in an -O-P or -N-P link, or 3. lipid impurities not completely extracted by the solvents.

In addition to the chloroform/methanol extractions illustrated previously in Plates II, III, IV, and V, the lipid pattern of an ether extract of component 6 is shown in P on these same plates. The lipid pattern seemed to be much the same as that for the chloroform/methanol extract. Not until the ether extracted component 6 was reextracted with the chloroform/methanol mixture was it possible to indicate the difference. As can be seen in Plate III, in the channels in which the chloroform/methanol extract of the ether extracted component 6 was spotted, no triglyceride or cholesterol ester was observed. Evidently these were completely removed by the non-polar solvent. The phospholipid was, however, not completely removed (Plate II). Comparing the quantity of lipid in the phospholipid spots of the ether extract and the spots of the subsequent chloroform/methanol extraction, it appears that about 80% of the phospholipid was extracted by the ether, and 20% remained behind after the ether extraction. Almost all of the residual phospholipid was later extracted by the chloroform/methanol mixture.

The nitrogen content of native component 6 was determined by the microkjeldahl method. By subtracting the amount of nitrogen present as phospholipid from the total nitrogen percentage and then multiplying the amount of nitrogen by 6.25, an estimate of the quantity of protein in component 6 was obtained. The native component 6 was found to contain about 5.41% protein. As previously mentioned, 60.9% of the molecule was lipid, and 33.7% of the molecule was water (see Table III). Dry component 6 was 91.9% lipid and 8.2% protein.

In order to shed further light on the relationship of this protein to the lipid and water portion of the lipoprotein, amino acid determinations were performed. These determinations were carried out by ion exchange chromatography as previously described.

The percentage of the original protein recovered from the column was estimated by means of several determinations; 1. a nitrogen determination by the kjeldahl method, 2. a determination for amide nitrogen, and 3. a colorimetric determination of tryptophan. Nitrogen determination by the kjeldahl method gave the percentage of total nitrogen in the sample. This includes the nitrogen present as amino acids, and nitrogen present as amides. In order to accurately determine the amount of protein applied to the column, the amount of nitrogen contributed to the total by amides and by tryptophan, which is destroyed by hydrolysis, must be subtracted. The chloroform/methanol extracted component 6 was found to contain 11.3% total nitrogen. Four tenths of one percent of this total was contributed by amide nitrogen, and 0.1% was found to be tryptophan. This left 0.108 mg. of nitrogen per mg. of delipidized component 6 which was contributed by the amino acids with the exception of tryptophan. These data are contained in Table III. This quantity of nitrogen was then multiplied by the factor 6.25 to obtain an estimate of the protein applied to the column. The amount of protein recovered from the column was calculated by multiplying the concentration of each amino acid as received from the column by its molecular weight minus the weight of one water molecule. Delipidized component 6 contains 0.675 mg. of protein present as amino acids (with the exception of tryptophan) per mg. of sample. This value was the amount of amino acid applied to the column. A total of 0.636 mg. of protein per mg. of sample was recovered from the column as amino acids. This is a 94.3% recovery.

Table IV summarizes the results of 20, 40, and 70 hours hydrolysates of delipidized component 6. Each value is the average of from 4 to 6 runs. To obtain the average value stated in the fourth column of numbers, the average was taken of the three hydrolysate periods, except in the case of serine, threonine, and arginine. These values were obtained by extrapolating to zero time. These three amino acids are partially destroyed during lengthened hydrolysis periods. On the other hand, valine-leucine and valine-isoleucine dipeptide bonds are not broken easily and these require a long hydrolysis period. To obtain these values, the 40 and 70 hour values are averaged. A graph of a typical run is shown in Graph I.

Values obtained by other authors (5,23) for the amino acid composition of similar proteins agree in the overall picture (see last column of Table IV), if not number for number. They find a high amount of aspartic and glutamic acid and leucine, as is also evident in component 6.

Margolis (26) compared the concentration of non-polar amino acids of soluble proteins, and structural proteins. As is shown in the following summary, he noted that structural lipoproteins had a higher content of these amino acids.

Protein	Mole % of Non-polar Amino Acids*
Lipid-free proteins	39.6%
Lipoproteins and proteolipids	46.8%
Structural lipoproteins	49.5%
Soluble lipoproteins	40.6%
Alpha-lipoproteins	37.6%
Beta-lipoproteins	41.7%
Component 6	44.2%

*Alanine, valine, leucine, isoleucine, phenylalanine, cystine, methionine, and proline.

He also pointed out that the quantity of these amino acids in serum beta-lipoproteins was close to the values for soluble lipoproteins. Component 6 has a

TABLE IV

Amino Acids	20 Hr. uM/mg.S*	40 Hr. uM/mg.S	70 Hr. uM/mg.S	Average uM/mg.S	Average $M/10^2$g.S	Average $M/10^2$g.P**	Values of Margolis $M/10^2$g.P(a)
Aspartic	0.554±0.052	0.519±0.047	0.577±0.055	0.550	55.0	81.5	82.1
Threonine	0.338±0.038	0.329±0.050	0.306±0.041	0.356	35.6	51.8	49.1
Serine	0.374±0.016	0.300±0.027	0.290±0.040	0.402	40.2	59.6	62.9
Glutamic	0.570±0.030	0.510±0.038	0.583±0.017	0.554	55.4	82.1	95.7
Proline	0.160±0.057	0.158±0.062	0.172±0.050	0.163	16.3	24.1	29.0
Glycine	0.250±0.014	0.229±0.036	0.282±0.021	0.253	25.3	37.5	37.2
Alanine	0.414±0.019	0.448±0.015	0.436±0.020	0.433	43.3	64.1	47.2
Cystine	0.060±0.008	0.060±0.007	0.065±0.010	0.062	6.2	9.2	5.1
Valine	0.370±0.023	0.449±0.017	0.452±0.036	0.450	45.0	66.7	47.3
Methionine	0.076±0.011	0.094±0.015	0.087±0.009	0.086	8.6	12.7	14.0
Isoleucine	0.332±0.020	0.353±0.041	0.392±0.024	0.372	37.2	55.1	47.2
Leucine	0.518±0.035	0.566±0.021	0.615±0.015	0.590	59.0	87.4	91.1
Tyrosine	0.229±0.040	0.216±0.037	0.197±0.051	0.214	21.4	35.6	22.9
Phe-Ala	0.239±0.042	0.208±0.051	0.246±0.045	0.231	23.1	34.2	40.7
Lysine	0.248±0.038	0.250±0.021	0.263±0.019	0.254	25.4	37.6	51.9
Histidine	0.047±0.012	0.045±0.008	0.035±0.010	0.042	4.2	6.2	19.4
Ammonia	0.385±0.043	0.443±0.057	0.507±0.046	0.495	49.5	73.3	107.2
Arginine	0.180±0.020	0.178±0.016	0.188±0.030	0.182	18.2	27.1	24.2

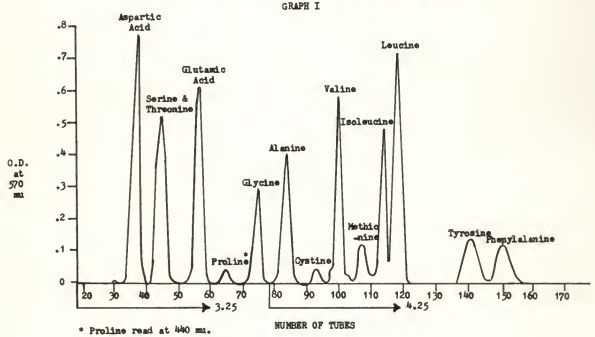
* S = Sample

**P = Protein

(a) = Values obtained by Margolis for a similar lipoprotein found in human serum. Values in the above table are averages of from 4 to 6 runs.

TYPICAL RUN OF 20 HOUR HYDROLYSATE OF DELIPIDIZED COMPONENT 6

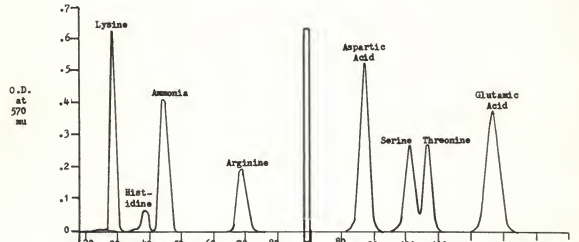
GRAPH I



* Proline read at 440 mμ.

1.48 mg. of component 6.

50 cm. column. Aminex A-4 (Acidic Column)



1.48 mg. component 6.

8 cm. column. Aminex A-5 (Basic Column)

1.48 mg. component 6.

150 cm. column. Amberlite IR-120 (Acidic Column)

concentration of non-polar amino acids which is closer to the values obtained for soluble lipoproteins.

The fact that the protein exhibits a high concentration of glutamic and aspartic residues, and a lower concentration of non-polar amino acids such as is found in the soluble lipoproteins, suggests perhaps, that a large portion of the binding properties is due to electrostatic forces. These forces would be possible salt linkages between phosphatidyl ethanolamine, and lecithin and the negatively charged group of the dicarboxylic acids.

Delipidized component 6 was found to contain an easily hydrolyzed nitrogen component which was assumed to be amide nitrogen. A total of 0.301 micromoles of this amide nitrogen was found per mg. of sample. (See Table III.) Since there are a total of 1.104 micromoles of dicarboxylic amino acids present per mg. of delipidized component 6 (see Table IV), this still leaves a large portion free to combine with the basic group of phospholipid. The free negative charges of the dicarboxylic acids could neutralize the positive charge of phosphatidyl ethanolamine and lecithin, thus forming a bond. Possible bonds such as these could account for the phospholipid which was not extracted by ether.

As was metnioned before, approximately 20% of the phospholipid could not be removed by extraction with ether. An attempt was made to correlate the number of phospholipid residues not extracted with ether to the number of free dicarboxylic acid residues. The total lipid of component 6 contains 0.008 mg. of phosphorous per mg. of lipid, or 0.258 micromoles of phospholipid per mg. of dry whole component 6. Twenty percent of this value is 0.056 micromoles of phospholipid per mg. of whole dry sample still present in the molecule after ether extraction. The amount of protein per mg. of dry whole component 6 is 0.033 mg. (Table III). In this amount of protein there are 0.085 micro-

moles of dicarboxylic acid. Of these, 0.030 micromoles are amide nitrogen. When the micromoles of dicarboxylic acid tied up by possible amide linkages were subtracted from the total micromoles of amino acid, a value of .055 micromoles of dicarboxylic amino acid was obtained. Thus the molar concentration of the free dicarboxylic amino acid was obtained. Thus the molar concentration of the free dicarboxylic amino acids is 0.056 and the concentration of the unextracted phospholipids is 0.055. The amount of unextracted phospholipid and the amount of the dicarboxylic acid residues are in the same range. This supports the idea that the phospholipid and the dicarboxylic acids may be bound. They may be bound tight enough to prevent the extraction of a considerable portion of the phospholipid with ether.

In summary, the protein moiety of component 6 was found to be like many soluble lipoproteins, having a lower concentration of non-polar amino acid residues than the structural lipoproteins. Twenty-one percent of the amino acid residues of component 6 were found to be dicarboxylic acid residues. The molar concentration of free dicarboxylic acid residues, and the molar concentration of phospholipid not extracted by ether agree quite closely. All of the triglyceride was extracted with ether. Almost 34% of the native molecule was found to be water, probably essential for the structural integrity of the lipoprotein. This water seems to be far too great a quantity to be carried simply as hydrated protein. Malik (25) has found that one of the three NH_2 terminal groups found in component 6 was masked by the lipid. Taking into account all these findings relative to component 6, it would appear that the model as proposed by Vandenheuval (50) may be used to describe the structure of component 6.

According to Vandenheuval (50), the center of this schematic diagram of a lipoprotein is water, next is a thin layer of phospholipid, next is a thick

layer of triglyceride with some cholesterol, and cholesterol esters. On the outside is a thin coil of protein, the N-terminal of which may or may not be embedded in the triglyceride under layer. To this hydrated protein shell, there is attached phospholipid, by means of salt linkages. The neutral fatty acid chains of these molecules extend out into the triglyceride layer. The inside of the triglyceride layer is covered by a shell of phospholipid, to make the internal environment favorable to water. The fatty acid chains of these are also extending out into the triglyceride environment. The triglyceride, cholesterol esters and cholesterol seem to be dissolved, or bound by hydrophobic bonding to these fatty acid chains, because of the ease of extractability with non-polar solvents alone. Especially the triglyceride seems to be bound this way, since the huge quantity of triglyceride present in the total molecule is completely extracted by cold ether.

In view of the fact that Malik (25) found three NH_2 terminal amino acid residues, and only one of them was masked by lipid, there appears that there may be more than one polypeptide moiety involved in the fraction. The methods used to determine the purity of component 6, i.e. electrophoresis, and the ultracentrifuge, showed no heterogeneity because the huge quantity of lipid the molecule carries would very greatly affect the density, and would make the molecule very large, causing it to move with difficulty through an electrophoretic medium.

Future work to determine further the structure of this large molecule may include partial extraction with N-heptane (17) or ether at very low temperatures. This might be instrumental in determining whether or not there are several distinct protein residues present in component 6. Extractions in this manner would leave the residue soluble, and a minimum of denaturation would have occurred. Also the size of the moiety would be considerably smaller and

could be more easily separated by such methods as disc electrophoresis. To further characterize the linkage between the lipid and the polypeptide moieties partial digestion with proteolytic enzymes might be useful. After part of the protein has been digested away, solubility studies, differential extractions with lipid solvents, and amino acid analyses of the residues may give added information about the structure of the molecule.

SUMMARY

The total lipid, protein, and water content of the native component 6 was determined by physical and chemical means. The lipid composition of native component 6 was determined by thin layer chromatographic techniques and chemical methods. Differential extraction of native component 6 with non-polar solvents was used to gain a possible insight into the association of lipid with the polypeptide portion of the molecule, as described earlier. Amino acid analysis by means of ion exchange column chromatography was performed by the method of Moore and Stein, and tryptophan and amide nitrogen were determined by chemical means stated previously.

It was observed that the total component 6 contained 60.9% lipid, 33.6% water, and 5.41% protein. The lipid portion of the native molecule contained approximately 20% phospholipid, 75% triglyceride, 4% cholesterol, and 1% cholesterol esters. The polypeptide portion of the molecule was found to have a high concentration of polar amino acids, and a very low concentration of sulfur containing amino acids.

It was also noted that 80% of the phospholipids in the native molecule were extracted with the non-polar solvent, leaving 20% behind. The molar concentration of the phospholipids left in component 6 after extraction compares very closely with the molar concentration of the dicarboxylic amino acid residues determined to be free in the polypeptide portion of the molecule.

From the foregoing observations, it could be stated that a relationship exists between the phospholipid of the lipid portion and the dicarboxylic acid residues of the peptide portion, which may be involved in linking the protein portion of the molecule with the peptide portion.

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LITERATURE CITED

(1) Anfinsen, C.B. Advances in Protein Chemistry. Vol. 20. Academic Press, New York and London. 1965.

(2) Applied Science Laboratories. Catalog No. 10. Spring, 1966.

(3) Avigan, J.J. Modifications of Human Serum Lipoprotein Fractions by Lipid Extraction. Jour. Biol. Chem. 226:957-964. 1957.

(4) Avigan, J.J., R. Redfield, D. Steinberg. N-Terminal Residues of Serum Lipoproteins. Bioch. et Biophys. Acta. 20:557-559. 1956.

(5) Ayrault-Jarrier, M., F. Martinet, and J. Polonovski. Action of Determents on an Alpha-Lipoprotein of Human Serum. II. Extractability of the Lipids. Bull. Soc. Chim. Biol. 45:369-376. 1963.

(6) Beckman Technical Bulletin. A-TB-007B. April, 1963.

(7) Bigland, C.H. Poor Serum Yields from Avian Blood as a Result of Delayed Clotting. Poultry Science. 43:4-6. July-November, 1964.

(8) Bio-Rad Laboratories. Aminex Resins, Buffers, Reagents for Amino Acid Analyses. Bulletin 115A4. March, 1966.

(9) Clegg, R.E., P.E. Sanford, R.E. Hein, W.C. Andrews, J.S. Hughes and C.D. Mueller. Electrophoretic Comparison of Two Serum Proteins of Normal and DESB Treated Cockerels. Science. 114:437-439. 1951.

(10) Clegg, R.E. and R.E. Hein. P^{32} Distribution in the Serum Proteins of the Chicken. Science. 117:714-715. 1953.

(11) Clegg, R.E., R.E. Hein, C.H. Suelter and R.H. McFarland. The Distribution of Radioactive Phosphorous in the Electrophoretic Components of Egg Yolk Proteins. Poultry Science. 34:210-214. 1955.

(12) Colowick, S.P., and N.O. Kaplan. Methods in Enzymology. Vol. 6. Academic Press, New York and London, 1963, pp. 819-822.

(13) Davis, B.J. Disc Electrophoresis II. Method and Application to Human Serum Proteins. Ann. N.Y. Acad. Sci. 121(2):404-27. 1964.

(14) deLalla, O., and J.W. Gofman. Methods of Biochemical Analysis. Vol. 1. Interscience Press, New York, N.Y., 1954, pp. 459-468.

(15) Flickinger, J., and R.A. Rounds, and D.E. Rounds. The Material Synthesis of Egg Yolk Proteins as Demonstrated by Isotopic and Serological Means. Biochim. Biophys. Acta. 22:38-46. 1956.

(16) Gustafson, A., P. Alaupovic, and R.H. Furman. Studies of the Composition and Structure of Serum Lipoproteins; Physical-Chemical Characterization of Phospholipid-Protein Residues Obtained from Very-Low-Density Human Serum Lipoproteins. Biochim. Biophys. Acta. 84:767-769. 1964.

(17) Gustafson, A., P. Alaupovic and R. Furman. Studies of the Composition and Structure of Serum Lipoproteins. Separation and Characterization of Phospholipid--Protein Residues Obtained by Partial Delipidization of Very-Low-Density Lipoproteins of Human Serum. Bioch. 5:632-640. February, 1966.

(18) Heim, W.G. and A.M. Schectman. Electrophoretic Analysis of the Serum of the Chicken During Development. Jour. Biol. Chem. 209:241-247. 1955.

(19) Hillyard, L.A., C. Entenman, H. Feinberg, and I.L. Chaikoff. Lipid and Protein Composition of Four Fractions Accounting for Total Serum Lipoproteins. Jour. Biol. Chem. 214:79-90. 1955.

(20) Infante, R., and J. Polonoski. Estrogen Induced and Lipid Biosynthesis in the Pullet. Epatologia. 9:404-18. 1963.

(21) Inglis, A.S., and I.H. Leaver. Studies in the Determination of Tryptophan, Modified Fischl Procedure. Analyt. Bio. 7:10-17. 1964.

(22) Lewis, L.A., A.A. Green, and I.H. Page. Ultracentrifuge Lipoprotein Pattern of Serum of Normal Hypertensive and Hypothyroid Animals. Amer. J. Physiol. 171:301-400. 1952.

(23) Lindgren, F.T., A.V. Nichols. The Plasma Proteins. Vol. 2. F. W. Putnam (ed.), Academic Press, New York, N.Y., 1960, pp. 252.

(24) Macheboeuf, M.A. The Physicochemical State of Lecithin and the Esters of Cholesterol in the Blood Serum and Plasma. Bull. Soc. Chim. 45:602-603. 1929.

(25) Malik, S.K. The Amino Acid Composition and NH_2-Terminal Amino Acids of Component 6 of Diethylstilbestrol Treated Cockerels. MS Thesis. Kansas State University, 1961.

(26) Margolis, S. and R.G. Langdon. Studies on Human Serum B_1-Lipoproteins. I. Amino Acid Composition. Jour. Biol. Chem. 241:(2) 469-477. 1965.

(27) Margolis, S. and R.G. Langdon. Studies on Human Serum B_1-Lipoproteins. II. Chemical Modifications. Jour. Biol. Chem. 241:(2) 477-485. 1965.

(28) Margolis, S. and R.G. Langdon. Studies on Human Serum B_1-Lipoproteins. II. Enzymatic Modifications. Jour. Biol. Chem. 241:(2) 485-493. 1965.

(29) Misra, U.K. Fractionation of High Phosphorous Containing Protein of Chicken Blood Serum. PhD. Thesis. 1959. Kansas State University.

(30) Moore, D.H. Effect of Reciprocal Steroid Treatment on the Electrophoretic Patterns of Fowl Sera. Endocrinology. 42:38-45. 1948.

(31) Moore, S., D.H. Spackman, and W.A. Stein. Chromatography of Amino Acids on Sulfonated Poly Styrene Resins. An Improved System. Anal. Chem. 30:(7) 185-190. 1958.

(32) Moore, S. and W.H. Stein. A Modified Ninhydrin Reagent for the Photometric Determination of Amino Acids and Related Compounds. Jour. Biol. Chem. 211: Nov.-Dec. 1954.

(33) Niederl, J.B. and V. Niederl. Organic Quantitative Microanalysis. John Wiley and Sons, Inc., New York, N.Y. 1946, pp. 69-77.

(34) Ornstein, L. Disc Electrophoresis I. Background And Theory. Ann. N.Y. Acad. Sci. 121(2):321-49. 1964.

(35) Oser, B.L. Hawk's Physiol. Chem. McGraw-Hill Book Company, New York, N.Y., 1965. pp. 1060.

(36) Polonovski, J. "Role of Plasma Proteins in Transport of Lipids". Transport and Function of Plasma Proteins. Paris, 1965. West-European Symposium on Clinical Chemistry. Vol. 5. Elsevier Pub. Co., Amsterdam, London, and New York, N.Y., 1966. pp. 45-55.

(37) Rodbell, M. N-Terminal Amino Acid and Lipid Composition of Lipoproteins from Chyle and Plasma. Science. 127:701-705. 1958.

(38) Rodbell, M. and D.S. Frederickson. Nature and Function of Chylomicron Proteins. Federation Proceedings. 17:298-311.

(39) Scanu, A., L.A. Lewis, F.M. Bumpus. Separation and Characterization of the Protein Moiety of Human Alpha$_1$-Lipoprotein. Archives of Bioch. and Biophys. 74:390-397. 1958.

(40) Scanu, A., L.A. Lewis and I. Page. The Antigenicity of Beta and Alpha$_1$-Lipoproteins of Human Serum. Jour. of Exptl. Med. 108:185-196. 1958.

(41) Schjeide, O.A. Lipoproteins of the Fowl - Serum, Egg and Intracellular. Progress in the Chemistry of Fats and Other Lipids. Vol. 6. Holman, Lundberg, and Malkin, Editors. 1963.

(42) Schjeide, O.A. and R.W. Dickinsen. Partition Cell for Spinco Preparative Ultracentrifuge. Analytical Chemistry. 25:999-1003. 1953.

(43) Schjeide, O.A. and M.R. Urist. Protein and Calcium in Serums of Estrogen-Treated Roosters. Science. 124:1242-1249. 1956.

(44) Schmidt, D.E. and W.S. Ruliffson. A Colorimetric Procedure for Determining Monoamine Oxidase Activity Based on Ammonia Evolution. Submitted for Editorial Review to Anal. Bioch. July, 1967.

(45) Shore, B. C- and N-Terminal Amino Acids of Human Serum Lipoproteins. Arch. Biochem. Biophys. 71:1-10. 1957.

(46) Shore, B. and V. Shore. Amino Acid Composition of the Protein of Human Serum Lipoproteins. Plasma. 2:621-625. 1954.

(47) Shore, V., and B. Shore. The Protein Subunit of Human Serum Lipoproteins of Density 1.125-1.200 g/ml. Bioch. et Biophys. Res. Comm. 9(5): 455-460.

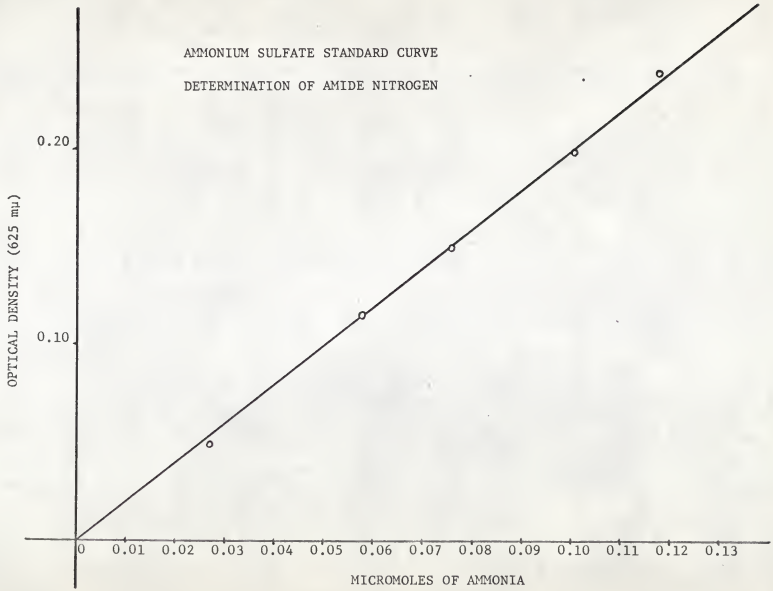
(48) Sperry, W.M. and F.C. Brand. The Determination of Total Lipids in Blood Serum. Jour. Biol. Chem. 213:69-73. 1955.

(49) Stahl, E. Thin Layer Chromatography. A Laboratory Handbook. Academic Press, New York and London. 1965. pp. 5-14.

(50) Vandenheuval, F.A. The Origin, Metabolism, and Structure of Normal Human Serum Lipoproteins. Canad. J. Bioch. Physiol. 40:1299-1320. 1962.

(51) Vanstone, W.E., W.G. Dale, W.F. Oliver and R.H. Common. Sites of Plasma Phosphoprotein and Phospholipid in the Estrogenized Cockerel. Canad. J. Bioch. Physiol. 35:659-665. 1957.

APPENDIX A


AMMONIUM SULFATE STANDARD CURVE
DETERMINATION OF AMIDE NITROGEN
OPTICAL DENSITY (625 mμ)
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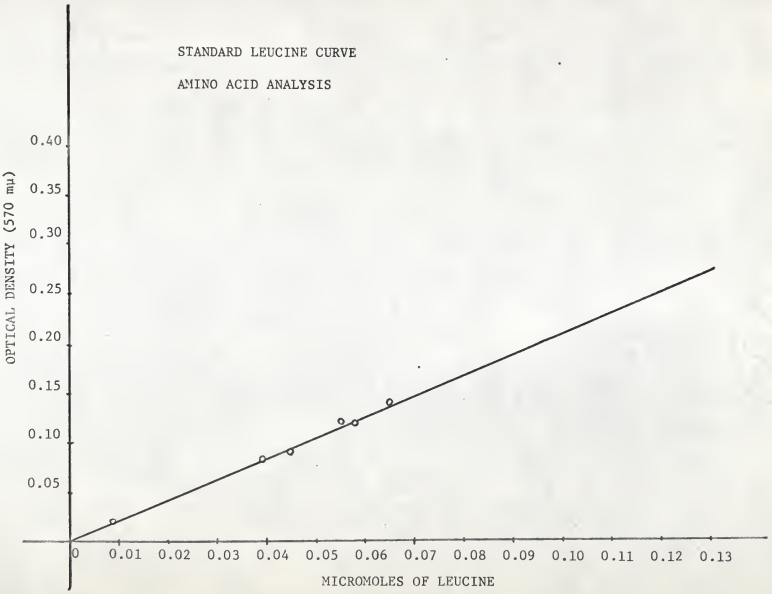
0.10
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
0.11
0.12
0.13
MICROMOLES OF AMMONIA

APPENDIX B


STANDARD LEUCINE CURVE
AMINO ACID ANALYSIS
OPTICAL DENSITY (570 mμ)
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0.35
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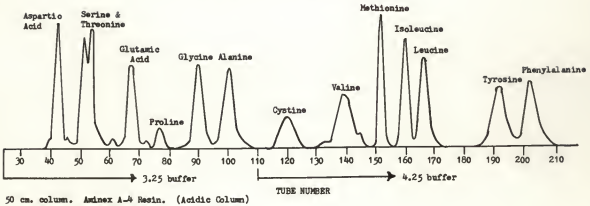
0.25
0.20
0.15
0.10
0.05
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
0.11
0.12
0.13
MICROMOLES OF LEUCINE

APPENDIX C

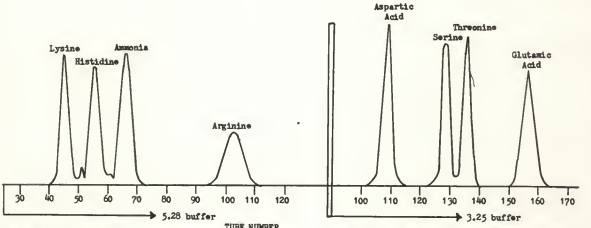
TYPICAL STANDARD RUN OF BECKMAN AMINO ACID MIXTURE



50 cm. column. Aminex A-4 Resin. (Acidic Column)

0.250 micromoles of amino acid

Absorbance: 570 lambda. Proline read at 440 lambda.



8 cm. Column. Aminex A-5 (Basic Column)

0.250 micromoles of amino acid

Absorbance: 570 lambda

150 cm. Column. Amberlite IR-120 (Acidic Column)

0.500 micromoles of amino acid

Absorbance: 570 lambda

STANDARD AMINO ACID RUNS

Amino Acid	Micromoles Recovered	% Recovery
Aspartic Acid	0.253 ± 0.009	100.7%
Serine*	0.493 ± 0.021	99.0%
Threonine*	0.489 ± 0.033	97.9%
Glutamic Acid	0.250 ± 0.010	100.0%
Proline	0.230 ± 0.023	92.1%
Glycine	0.245 ± 0.009	98.0%
Alanine	0.251 ± 0.013	100.4%
Cystine	0.237 ± 0.025	94.7%
Valine	0.253 ± 0.007	101.1%
Methionine	0.246 ± 0.014	98.4%
Isoleucine	0.250 ± 0.009	100.0%
Leucine	0.248 ± 0.017	99.2%
Tyrosine	0.249 ± 0.013	99.6%
Phenylalanine	0.245 ± 0.013	98.0%
Lysine	0.253 ± 0.012	101.1%
Histidine	0.247 ± 0.015	98.7%
Ammonia	0.250 ± 0.009	100.0%
Arginine	0.243 ± 0.011	97.2%

Sample size introduced was 0.250 micromoles of each amino acid. This was a standard mixture supplied by Beckman; Beckman Mixture No. 1.

*Sample size for Serine and Threonine, which were run in a different column from the other amino acids, was 0.500 micromoles each.

Six runs were made to obtain the above averages, except for Serine and Threonine for which 4 runs were made.

A CHARACTERIZATION OF COMPONENT VI, A LIPOPROTEIN, FOUND IN THE BLOOD SERUM OF DIETHYLSTILBESTROL TREATED BIRDS

by

RAE MARIE DODGE

B. S., Kansas State University, 1965

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1968

The lipoproteins of serum consist of a polypeptide component in combination with lipid. This lipid includes phospholipid, cholesterol, cholesterol esters, and triglycerides. Previously, it was thought that the lipid and protein moiety were held mainly by hydrophobic bonding; however, recent work has revealed that a salt linkage possibly plays a large role. These linkages involve the free -COOH group of the dicarboxylic acid residues of the protein and phospholipid. In order to investigate possible linkages of this type in component 6, lipid analyses, differential extractions, and amino acid analyses were performed.

The serum of White Rock and Leghorn cockerels injected with diethylstilbestrol was used. The initial isolation of component 6 was done by means of the ultracentrifuge. The lipid composition of component 6 was determined by thin layer chromatography using four different solvent systems and by using standard lipid mixtures developed for these systems. Physical means were used to determine the total lipid content and chemical methods were used to determine cholesterol and phospholipid content. Differential extraction with cold ether was carried out in order to further characterize the lipid portion of the molecule.

After total delipidization of component 6 using 2:1 chloroform/methanol, an amino acid analysis on the polypeptide portion of the molecule was performed by the method of Moore and Stein.

It was observed that in the whole component 6, there was 60.9% lipid, 33.6% water and 5.41% protein. The lipid portion of the molecule contained 75% triglyceride, 20% phospholipid, 4% cholesterol, and 1% cholesterol ester. The differential extraction with cold ether showed that 20% of the phospholipid was not removed. Amino acid analysis revealed a high concentration of dicarboxylic amino acid residues, and almost no sulfur containing amino acids.

It was also noted that the molar concentration of the free dicarboxylic amino acid residues compared very closely with the molar concentration of the phospholipid residues not extracted with cold ether.

From the foregoing observations it was concluded that the phospholipid residues and the dicarboxylic acid residues of component 6 may be instrumental in the linkage of the lipid to protein, as proposed for human serum lipoproteins by several groups of workers.